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OSMOTIC RESPONSES DEMONSTRATING THE EXTRACELLULAR CHARACTER OF THE SARCOPLASMIC RETICULUM

BY R. I. BIRKS AND D. F. DAVEY*
From the Department of Physiology, McGill University, Montreal, Canada

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SUMMARY

1. Changes in the dimensions of the sarcoplasmic reticulum in frog sartorius muscles exposed to hypertonic and hypotonic solutions have been studied with the electron microscope.

2. The volume of the sarcoplasmic reticulum has been found to be linearly related with a negative slope to the reciprocal of the osmotic pressure. Over the range 0·75 to 3·5 × normal osmotic pressure the reticulum volume has been calculated to change from 11·5 to 18·5 % of normal cell volume.

3. These changes in sarcoplasmic reticulum volume correspond to the calculated changes in the volume of the intra-fibre sucrose compartment, postulated by earlier workers on the basis of studies on changes in cell volume with changes in osmotic pressure in living muscles.

4. To explain these and other related findings on the distribution of electrolytes in muscle, it is proposed that the sarcoplasmic reticulum of skeletal muscle is an extracellular compartment.

5. The significance of this hypothesis for the mechanism of excitation-contraction coupling is discussed.

INTRODUCTION

Dydyńska & Wilkie (1963) have shown in frog skeletal muscle exposed to hypertonic solutions that the volume of fibre water, calculated from weight and sucrose space determinations, decreases as if the cells were perfect osmometers. Cell volume, when determined by an optical method, did not decrease to the same extent in the hypertonic media. Although later more refined optical studies by Blinks (1965) cast doubt on the accuracy of the original method, they nevertheless confirmed the existence of this dis-

* Holder of a Muscular Dystrophy Association of Canada Fellowship.

crepancy. A possible explanation of this discrepancy was that part of the space measured with sucrose might lie within the muscle fibres and might enlarge in muscles exposed to hypertonic solutions (cf. Johnson & Simonds, 1962). Such a space would be included in optical measurements of cell volume but would be excluded from the measurements using sucrose. Electron microscopic examination by Huxley, Page & Wilkie (1963) revealed that some elements of the sarcoplasmic reticulum increased in volume in hypertonic solutions despite the overall shrinkage of the cells; however, the swelling was not uniformly distributed over the fibres, and it was not of sufficient magnitude to account for the discrepancy as measured by Dydyńska & Wilkie (1963).

The hypothesis that sucrose may enter the sarcoplasmic reticulum remains an attractive one, particularly if it may also be taken to indicate a more general accessibility of the reticulum to normal extracellular solutes. We have therefore re-examined the behaviour of the sarcoplasmic reticulum in muscles exposed to solutions of both increased and decreased osmotic strength using better fixation procedures than were available to the original workers and less extreme changes in tonicity. We now find that the reticulum increases in volume when the osmotic strength of the bathing medium is increased. The increases occur more or less uniformly throughout the fibres and include all elements of the reticulum. Comparison of our data with the volume data of Dydyńska & Wilkie (1963) and the optical data of Blinks (1965) suggests that expansion of the sarcoplasmic reticulum can account for the differences in cell volume noted above. Since no other cellular compartment appears to enlarge under these conditions our data suggest that the sarcoplasmic reticulum is accessible to sucrose. In the light of this conclusion, earlier studies (see Discussion) on the kinetics of movements of solutes in skeletal muscle may be interpreted to suggest that the sarcoplasmic reticulum is also accessible to many of the constituents of extracellular fluid, and thus may normally contain extracellular fluid or some modification of it.

METHODS

Solutions. The normal Ringer solution (denoted N Ringer) pH 7·0 contained: 115 mM-NaCl, 2·5 mM-KCl, 1·8 mM-$CaCl_2$, 2·15 mM-Na_2HPO_4, and 0·85 mM-$NaH_2PO_4.H_2O$. Solutions of increased or decreased tonicity were prepared by using 'a' × 115 mM-NaCl to obtain Ringer tonicity 'a' × Normal (denoted 'a' × N Ringer): e.g. 1·5 × N Ringer contained 172·5 mM-NaCl. 1·5 × N sucrose-Ringer was prepared by adding 105 mM sucrose to N Ringer producing a solution isosmotic to 1·5 × N Ringer, but with normal electrolyte concentrations. When solutions with tonicities greater than 2 × Normal were prepared, the ratio of primary to secondary sodium phosphate was altered to maintain pH 7·0.

Experimental procedure. Sartorius muscles from *Rana temporaria* were excised, and mounted spirally at rest length on 5 mm diameter Perspex rods. They were then placed in the experi-

mental solution for the required time, and fixed on the rods. Control muscles, obtained in most experiments from the opposite legs of the same frogs, were soaked in Ringer solution for the same times, and fixed in the same way. Two to three muscles were used for each control and test.

Fixation. For most of the experiments the muscles were fixed for 30 min at 0° C in acrolein (6%, v/v; Sabatini, Bensch & Barrnett, 1963) in phosphate buffer (100 mM, pH 7·4) containing 1 mM-$CaCl_2$, then washed for 15 min in this same buffer. This procedure was followed by 30 min post-fixation at 0° C in osmium tetroxide (1%, w/v) buffered to pH 7·4 with veronal acetate (Palade, 1952). In one experiment this latter solution was used for primary fixation and acrolein was not used. In a further experiment the osmotic strength of the acrolein solution used to fix the muscles exposed to 1·5 × N Ringer was increased by the addition of 110 m-osmole sucrose (38 g/l.). After fixation the muscles were washed for 30 min in several changes of the veronal acetate buffer at room temperature, during which time small blocks suitable for embedding were cut from the thin borders of the muscle. The blocks were stained by soaking for 45 min in uranyl acetate (0·5%, w/v; Watson, 1958) buffered to pH 5 with veronal acetate at room temperature, and then dehydrated in graded ethanol and embedded in Epon (Luft, 1961).

Microscopy. Sections exhibiting silver-grey interference colours were mounted on carbon (Watson, 1955) or silicon monoxide-coated grids, stained with lead citrate (Venable & Coggeshall, 1965) and examined in a Siemens Elmiskop I electron microscope at 18,500 × magnification.

Measurement of volume changes. The sarcoplasmic reticulum in frog skeletal muscle is longitudinally oriented and curves around the myofibrils. Longitudinal sections provide information mainly on the longitudinal dimensions of the sarcoplasmic reticulum, whereas cross-sections yield accurate information on the transverse dimensions. Preliminary experiments indicated that the most prominent effects of changes in the tonicity of the extracellular fluid were exerted on the transverse dimensions of the sarcoplasmic reticulum. In the present experiments therefore, cross-sections were chosen for measurement of reticular volume changes. Micrographs were obtained with planes of section passing through the three main reticular zones: the H-zone sacs (fenestrated collar, Peachey, 1965), the longitudinal tubules, and the triad sacs (terminal cisternae). The intermediate cisternae described by Peachey were not prominent in the muscles we have examined. All tubular elements between the H-zone sacs and the triad sacs were therefore treated as longitudinal tubules.

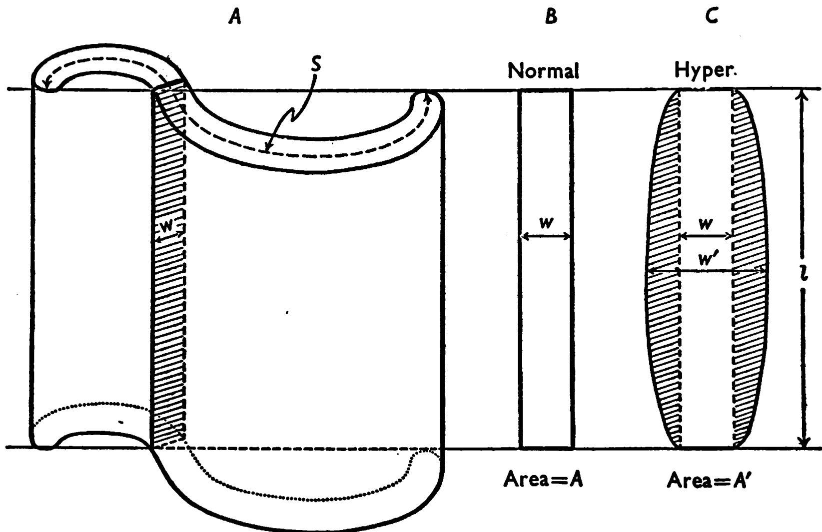
In calculating the volume of the sarcoplasmic reticulum, this slightly different distribution of its volume over the sarcomere necessitated a modification to the estimated volume distribution of Peachey (1965). We have estimated that the reticulum occupies the following fractions of cell volume: H-zone sacs, 3·5%; longitudinal tubules, 3·5%; and triad sacs, 6%, and that the triad sacs contain solid matter equal to approximately one half their volume. Thus the solid matter of the triad sacs is estimated to be 3% of cell volume.

To permit statistical treatment of the results it was desirable to measure dimensions of the reticulum in cross-section that were least variable in control muscles, and most sensitive to volume changes in experimental muscles. There is considerable variability in the form of the reticular profiles observed in different cells, particularly between cells exposed to different tonicities. This is especially true of the H-zone sacs, which appear in cross-section as segments of an annulus surrounding each myofibril. The degree of segmentation is highly variable. We found however that the width, measured in a direction normal to the plane separating adjacent myofibrils, was most sensitive to volume changes. For these reasons the widths, or short axes of the H-zone and triad sacs, and the diameters of the longitudinal tubules have been used as the index of volume changes in the reticulum.

For each of the above mentioned zones, fifteen to twenty-five micrographs from cells with no signs of damage, from both experimental and control muscles, were used for the measurements of the reticulum in each experiment. Each group of micrographs was obtained from

at least three control or experimental tissue blocks. Since the same muscle fibre did not provide pictures of each zone measured, on the average the data for each control and test experiment represent samples from about fifty different muscle fibres in two to three different muscles. All sacs of the appropriate type in each micrograph were measured to avoid any possible bias on the part of the experimenters. In addition, where dimension changes were not visually obvious, experimental and control micrographs were mixed and the measurements were performed blind. Dimensions were obtained to the nearest 100 Å, usually to the nearest 0·7 mm on the photographic prints.

To provide an index of changes in over-all cell volume, the mean spacing of myosin filaments was measured (Huxley *et al.* 1963). Statistical information was prepared with the aid of an IBM 7090 computer. Results are presented as the mean ± standard error of the mean.



Text-fig. 1. Diagrammatic representation of H-zone and triad sacs to illustrate the method of estimating volume changes. For explanation see text.

Calculation of reticulum volume. The volumes of the H-zone and triad sacs were calculated from the measured dimensions in the following way. It was assumed that the sac volume is given by the product of the curvilinear dimension S (see Text-fig. 1*A*) and the mean area A, of a longitudinal section passing through the sac with its plane normal to the dimension S at the point of section (shaded area, Text-fig. 1*A*). It was also assumed that in solutions of normal osmotic pressure the area A can be represented by a rectangle of width w, the dimensions measured in the experiment presented here, and of undetermined length l, as shown in Text-fig. 1*B*. It was further assumed that in muscles exposed to hypertonic media in which the short axes have increased to w' due to reticular swelling, the shape of the section is no longer rectilinear, but rather can be approximated by the form indicated in Text-fig. 1*C*, in which the increased area (shaded) is composed of two hemi-ellipses having semi-axes $\frac{1}{2}l$ and $\frac{1}{2}(w'-w)$. The choice of an elliptical, rather than a linear or circular profile was made in order to coincide best with the appearance of reticular profile seen in longitudinal sections of muscles exposed to hypertonic media. On this basis the volume of the H-zone or triad sacs in muscles exposed to hypertonic solutions, V', is given by the equation

$$\frac{V'}{V} = 1+\frac{\pi}{4}\left(\frac{w'-w}{w}\right),$$

where V is the volume at normal osmotic pressure. The volume occupied by the longitudinal tubules was calculated by assuming that the volume of each tubule can be approximated to a cylinder undergoing diameter changes only.

RESULTS

Effects of hypertonic solutions

Effects of increasing osmotic pressure to $1 \cdot 5 \times$ *normal with NaCl.* In two experiments (eight muscles) in which the preparations were soaked for 10 min in $1 \cdot 5 \times N$ Ringer solution, and then fixed in acrolein, the mean myosin filament spacing decreased by 5 ± 2 % and 4 ± 1 % relative to control muscles soaked for 10 min in N Ringer (Table 2, Expts. 1 and 2), indicating that the cells had shrunk upon exposure to the hypertonic medium. The H-zone sacs and the longitudinal tubules of the sarcoplasmic reticulum underwent marked swelling when the cells shrank (Pl. 1): the mean short axis of the H-zone sacs in the two experiments increased by 94 ± 7 % and 70 ± 10 %, and the mean diameter of the longitudinal tubules increased by 21 ± 2 % and 18 ± 4 % (Table 1, Expts. 1 and 2).

In a third experiment, in which the soaking time was reduced to 1 min, the mean short axis of the H-zone sacs increased by 88 ± 10 % and the diameter of the longitudinal tubules increased by 19 ± 3 % (Table 1, Expt. 3). The rapidity with which these changes occurred is not surprising because the cells themselves had apparently adjusted to the change in osmotic pressure within this 1 min period as judged by the reduction in myosin spacing (Table 2, Expt. 3).

The triad sacs of muscles soaked for 10 min in $1 \cdot 5 \times N$ Ringer (Pl. 2) were less swollen than the other regions of the reticulum: the sac short axis increased by 12 ± 3 % and 10 ± 3 % in the two experiments (Table 1, Expts. 1 and 2), and by 18 ± 3 % (Table 1, Expt. 3) when the soaking time was 1 min.

In each of these three experiments, the difference in width of each zone of reticulum in the control and experimental muscles was significant at better than the 1 % level of probability (Table 1). Furthermore, the fractional changes in the reticulum dimensions and in the myosin filament spacings were very similar for the three experiments. However, the width of each region of the reticulum (Table 1), and the spacing of myosin filaments (Table 2) in control muscles varied somewhat from experiment to experiment. Since each experiment was performed on a different batch of frogs at a different time of the year, these variations are not surprising. Nevertheless, the pooled data for the three experiments also showed that the changes in reticulum dimensions in the hypertonic media were significant at better than the 1 % level of probability (Table 1). Thus the variations

TABLE 1. Changes in dimensions of the sarcoplasmic reticulum in anisotonic solutions

Expt. no.	Experimental Ringer solution	Soak time (min)	Fixative	Region measured	Mean measured dimension* Control (mμ)	Experimental (mμ)	Change (%)	P
1	1·5 × N	10	Acrolein	H-zone sacs	35 ± 1 (184)	69 ± 2 (190)	+94 ± 7	< 0·01
				Longitudinal tubules	41 ± 1 (825)	49 ± 1 (548)	+21 ± 2	< 0·01
				Triad sacs	136 ± 2 (131)	152 ± 2 (176)	+12 ± 3	< 0·01
2	1·5 × N	10	Acrolein	H-zone sacs	39 ± 2 (68)	66 ± 2 (140)	+70 ± 10	< 0·01
				Longitudinal tubules	34 ± 1 (289)	41 ± 1 (139)	+18 ± 4	< 0·01
				Triad sacs	107 ± 2 (127)	118 ± 2 (97)	+10 ± 3	< 0·01
3	1·5 × N	1	Acrolein	H-zone sacs	45 ± 2 (29)	85 ± 3 (68)	+88 ± 10	< 0·01
				Longitudinal tubules	46 ± 1 (223)	55 ± 1 (190)	+19 ± 3	< 0·01
				Triad sacs	111 ± 2 (81)	130 ± 2 (59)	+18 ± 3	< 0·01
Pooled data Expts. 1–3				H-zone sacs	37 ± 1 (281)	71 ± 1 (398)	+89 ± 6	< 0·01
				Longitudinal tubules	40 ± 1 (1337)	49 ± 1 (877)	+22 ± 2	< 0·01
				Triad sacs	119 ± 1 (339)	139 ± 2 (332)	+16 ± 2	< 0·01
4	1·5 × N (sucrose)	10	Acrolein	H-zone sacs	46 ± 1 (50)	78 ± 3 (61)	+69 ± 9	< 0·01
				Longitudinal tubules	46 ± 1 (577)	50 ± 1 (410)	+8 ± 2	< 0·01
				Triad sacs	133 ± 1 (182)	140 ± 2 (136)	+6 ± 2	< 0·01
5	1·5 × N	10	Osmium	H-zone + A band profiles	43 ± 2 (109)	72 ± 6 (211)	+67 ± 14	< 0·01
				Triad sacs	128 ± 2 (246)	133 ± 1 (194)	+4 ± 2	0·03
6	1·5 × N	10	Acrolein + sucrose	H-zone sacs	35 ± 1 (167)	65 ± 1 (403)	+88 ± 5	< 0·01
				Longitudinal tubules	43 ± 1 (268)	50 ± 1 (717)	+16 ± 2	< 0·01
				Triad sacs	122 ± 2 (110)	134 ± 2 (176)	+10 ± 2	< 0·01
7, 8, 9	3·5 × N†	10	Acrolein	H-zone sacs	46 ± 1 (167)	102 ± 4 (112)	+121 ± 11	< 0·01
				Longitudinal tubules	47 ± 1 (579)	56 ± 2 (181)	+20 ± 5	< 0·01
				Triad sacs	128 ± 2 (154)	146 ± 5 (164)	+14 ± 5	< 0·01
10	0·75 × N	10	Acrolein	H-zone sacs	49 ± 1 (115)	34 ± 1 (130)	−30 ± 2	< 0·01
				Longitudinal tubules	50 ± 1 (246)	46 ± 1 (277)	−8 ± 2	< 0·01
				Triad sacs	150 ± 2 (90)	147 ± 2 (98)	−2 ± 2	0·32

* Expressed as mean ± S.E. of mean. Bracketed values give number of observations.

† Pooled data from three experiments.

TABLE 2. Changes in spacing of myosin filaments in anisotonic solutions

Expt. no.	Experimental Ringer solution	Soak time (min)	Fixative	Measured dimension* Control (Å)	Measured dimension* Experimental (Å)	Change (%)	P
1	1·5 × N	10	Acrolein	350 ± 3 (46)	334 ± 5 (40)	−5 ± 2	< 0·01
2	1·5 × N	10	Acrolein	348 ± 4 (65)	334 ± 3 (69)	−4 ± 1	< 0·01
3	1·5 × N	1	Acrolein	356 ± 4 (18)	337 ± 4 (18)	−5 ± 2	< 0·01
4	1·5 × N (sucrose)	10	Acrolein	338 ± 3 (32)	324 ± 3 (55)	−4 ± 1	< 0·01
5	1·5 × N	10	Osmium	350 ± 5 (33)	334 ± 4 (50)	−5 ± 2	< 0·01
7, 8, 9	3·5 × N	10	Acrolein	358 ± 3 (59)	316 ± 4 (60)	−12 ± 1	< 0·01
10	0·75 × N	10	Acrolein	365 ± 3 (72)	377 ± 3 (72)	+3 ± 1	< 0·01

* Expressed as mean ± S.E. of mean. Bracketed values give number of observations.

between different batches of frogs, and from other possible sources of random variation, were considerably less than the variation introduced by treatment in the hypertonic solution.

Effect of increasing osmotic pressure to $1{\cdot}5 \times$ *normal with sucrose.* In a further experiment on a total of four muscles, the experimental preparations were soaked for 10 min in $1{\cdot}5 \times$ N sucrose-Ringer (see Methods) to discover whether the reticulum volume changes were due to the osmotic, rather than to the ionic changes in the bathing solutions. The myosin filament spacing in muscles exposed to $1{\cdot}5 \times$ N sucrose-Ringer was reduced by 4 ± 1 % in comparison with the spacing found in untreated controls, as it had been in $1{\cdot}5 \times$ N Ringer (Table 2, Expt. 4). The mean short axis of the H-zone sacs increased by 69 ± 9 % and the diameters of the longitudinal tubules and the short axes of the triad sacs increased by 8 ± 2 % and 6 ± 2 % respectively, all slightly less than in experiments using $1{\cdot}5 \times$ N Ringer (see Table 1, Expt. 4). In these muscles there was noticeable damage to the reticulum in a number of fibres, and these were not used for sampling purposes. However, it was observed that the intact reticulum in the damaged cells was considerably more swollen than in the intact ones.

Effects of fixative on reticulum swelling in hypertonic media. Huxley *et al.* (1963) examined muscles that had been soaked for 2 hr in $3{\cdot}5 \times$ N Ringer, then fixed in osmium tetroxide. They reported swelling of one or more of the triadic elements of the sarcoplasmic reticulum at the Z line and marked shrinking of the A band sacs. Since the results reported here, from muscles soaked for 10 min in $1{\cdot}5 \times$ N Ringer then fixed in acrolein were qualitatively quite different from those of Huxley *et al.* (1963), it was decided to determine whether the changes in $1{\cdot}5 \times$ N Ringer were independent of the fixative employed. Thus in one experiment on a total of four muscles, only osmium fixation was employed. Despite the precaution of photographing only the most well preserved cells micrographs containing intact H-zone sacs were rarely obtained, since preservation of sarcoplasmic reticulum is generally not as good with osmium fixation as it is with fixation by aldehydes, the typical difference being the tendency of tubules and cisternae to vesiculate. All H-zone reticulum profiles therefore were measured as if they were longitudinal tubules. For this reason the lumped data of the A band and H-zone sacs of Expt. 5, Table 1, are not strictly comparable with those of other experiments; however, the reticulum was still clearly distended. Furthermore, the myosin filament spacing in the control muscles, although slightly larger than in muscles fixed with acrolein (Table 2), showed the same percentage decrease of 5 ± 2 % when the muscles were exposed to $1{\cdot}5 \times$ N Ringer. The diameter of the lumped groups increased by 67 ± 14 %, a value between the factor of increase of the H-zone sacs and the longitudinal tubules in the experiments using

acrolein fixation. The triad sacs underwent a 4 ± 2% increase in short axis, only significant at the 3% level, presumably also attributable to the poor fixation with osmium.

Independence of reticulum swelling in hypertonic solutions on the osmotic strength of the fixative solution. The similarity of the reticulum swelling in muscles exposed to hypertonic media observed after fixation in acrolein with an osmotic strength of 1170 m-osmole (measured cryostatically) and in osmium tetroxide fixative with an osmotic strength of only 145 m-osmole suggests that the swelling of the reticulum occurred before fixation, and was not produced by an osmotic transient during the initial stages of fixation. The considerable difference in osmotic pressures of these fixatives points out the difficulty of determining what osmotic effect these compounds exert across biological membranes during the fixation process. It would seem that the osmotic effect of acrolein must be only a small fraction of the value determined cryostatically, and, that as fixation takes place any osmotic effects of the fixative solution may be primarily exerted through the buffer system.

One experiment was performed in which the osmotic pressure of the buffer system, used to fix the muscles exposed to $1{\cdot}5 \times$ N Ringer, was increased by 50% in order to confirm that osmotic transients occurring during fixation were not responsible for the reticulum swelling observed after fixation. Three muscles were soaked in N Ringer for 10 min and fixed in the usual acrolein fixative. Three muscles from the opposite legs of the same frogs were soaked for 10 min in $1{\cdot}5 \times$ N Ringer and then fixed in acrolein fixative to which had been added sucrose, 38 g/l. As shown in Table 1, this increase in the osmotic strength of the fixative had no effect on the amount of swelling of the reticulum caused by the exposure to $1{\cdot}5 \times$ N Ringer. The increase in dimensions of the H-zone sacs was 88 ± 5%, within the range (70–94%) found in the three experiments using the standard acrolein fixative. The longitudinal tubules in the experiment increased in diameter by 16 ± 2% and the triad sacs increased by 10 ± 2%. These values were again within the range of the earlier experiments (18–21% for the longitudinal tubules and 10–18% for the triad sacs). A similar lack of effect of the osmotic strength of osmium tetroxide fixative solutions on reticulum changes in muscles exposed to hypertonic solutions was found by Huxley, Page & Wilkie (1963).

Effects of increasing osmotic pressure to $3{\cdot}5 \times$ *normal with NaCl.* The observations of Huxley *et al.* referred to earlier, on muscles exposed for 2 hr to $3{\cdot}5 \times$ N Ringer are not what would be expected on the basis of the present findings in less hypertonic solutions, especially since the results reported here were similar whether acrolein or osmium fixation was used. We have, therefore, studied the effects of $3{\cdot}5 \times$ N Ringer on the sarco-

plasmic reticulum, but we have restricted the soaking period to 10 min and used acrolein rather than osmium as the fixative.

In three experiments on a total of ten muscles we found that after 10 min in 3·5 × N Ringer the fibre volume was considerably less than in 1·5 × N Ringer as judged by the change in myosin filament spacing. The reduction amounted to 12 ± 1 %, from a control of 358 Å (Table 2) as compared with a 16 % reduction from a control of 354 Å in the experiments of Huxley *et al.* (1963). The swelling of the reticulum was considerably greater after 10 min exposure to 3·5 × N Ringer than it had been after exposure to 1·5 × N Ringer. The H-zone sacs of the sarcoplasmic reticulum were grossly swollen in comparison with those of normal muscles (Pl. 3, fig. 1), the increase being 121 ± 11 % (Table 1). The increase in the longitudinal tubules was 20 ± 5 % and in the triad sacs it was 14 ± 5 % (Pl. 3, and Table 1). These results fit in well with those obtained when muscles were soaked in 1·5 × N Ringer.

We did not observe in these experiments any shrinkage of the H-zone sacs of gross swelling of any element of the triad system, as reported by Huxley *et al.* (1963). Furthermore, examination of longitudinal sections of these muscles revealed the persistence of connexions between the H-zone sacs and the triad sacs (see Pl. 4, fig. 1) whereas Huxley *et al.* had reported this continuity to be disrupted. However, there were in most of the muscles examined a number of fibres showing varying degrees of damage to the reticulum.

Plate 4, fig. 2 shows a cross-section through a damaged fibre from a muscle soaked in 3·5 × N Ringer for 10 min. The H-zone sacs are extremely dilated, and some of the reticular membranes are disrupted. In one such disrupted area (between the arrows in Pl. 4, fig. 2) there is a space between the adjacent myofibrils. The outline of this space, with the two isolated vesicles of reticulum lying within it, suggests that the reticulum may have occupied the entire space before breaking into the two vesicles. This suggestion is supported by the fact that interfibrillar spaces of this size are only infrequently seen in muscles without reticular damage. Occasionally, large areas of the cells were observed in which a substantial proportion of the sarcoplasmic reticulum had undergone damage of this form.

Reversibility of reticulum swelling. We examined this question of reticulum damage further because it did not appear consistent with the observations of Hodgkin & Horowicz (1957) and Howarth (1958) that the suppression of the contractile response of frog skeletal muscle which occurs upon exposure to 3·5 and 2·5 × N Ringer, is rapidly reversed when the muscles are returned to N Ringer. It seemed possible that the swelling of the reticulum in the grossly hypertonic media might render it particularly sensitive to damage by fixation. Such an explanation might also

explain why, with the more prolonged exposure to the hypertonic solutions and the poorer preserving characteristics of the osmium fixative used by Huxley *et al.* (1963), even more profound damage occurred. We therefore compared the preservation of sarcoplasmic reticulum in muscles soaked for 10 min in 3·5 × N Ringer before fixation, with the preservation in muscles which were returned to N Ringer for 10 min after a 10 min exposure to 3·5 × N Ringer before fixation. The latter group of muscles showed no signs of damage to the reticulum and they were in no obvious way distinguishable from control muscles soaked 20 min in N Ringer.

The simplest explanation of these findings is that fixation does not consistently preserve the distended membranes of the sarcoplasmic reticulum when the system is grossly swollen by solutions of greatly increased osmotic pressure, and that this problem is more severe with osmium fixation than with acrolein fixation.

Effects of hypotonic solutions

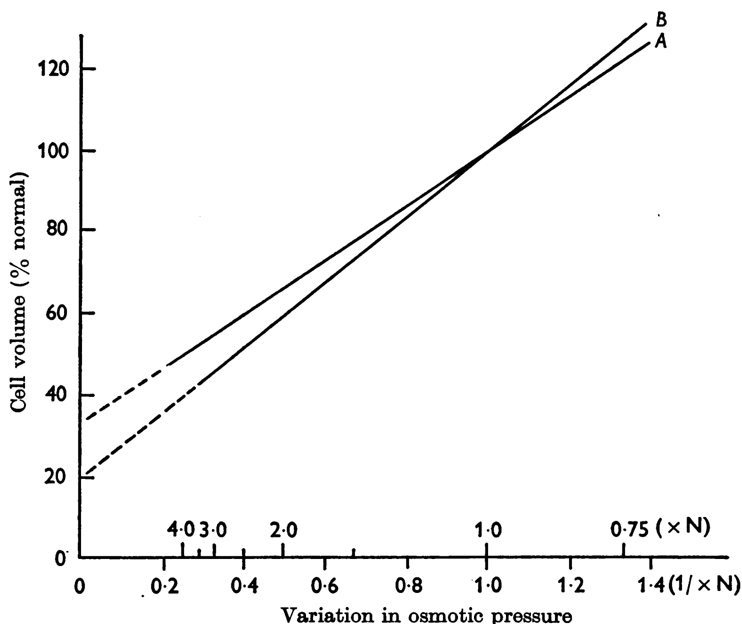
Effect of reducing osmotic pressure to 0·75 × *normal.* Four muscles were soaked for 10 min in 0·75 × N Ringer, fixed in acrolein, and the dimensions of the sarcoplasmic reticulum compared with the dimensions of reticulum in muscles exposed to N Ringer for a similar period. It was found that the reticulum had shrunk as a result of the treatment. As illustrated in Pl. 5 in some areas the opposing membranes of the H-zone sacs were almost touching as a result of the volume decrease. The mean decrease in short axis of the H-zone sacs was 30 ± 2 % (Table 1, Expt. 10), while the myosin filament spacing was increased by 3 ± 1 %. The decrease in the mean short axis of the triad sacs was only 4 ± 2 % and this change was significant only at the 32 % level of probability.

DISCUSSION

The main finding of this work is that changes in the dimensions of the sarcoplasmic reticulum induced by exposure of the muscles to anisotonic solutions occur in the opposite direction to the changes in volume of the cells themselves. These reticulum changes were independent of the fixative used, they were not affected by changes in the osmotic strength of the fixative solution, and they were readily reversible. Thus it appears reasonable to conclude that the volume of the sarcoplasmic reticulum in the living tissue increases with increasing environmental osmotic pressure. We would now like to examine whether the changes in sarcoplasmic reticulum volume, calculated from our data, fit with the changes in volume that would be predicted from the data of Dydyńska & Wilkie (1963) and Blinks

(1965) were the sarcoplasmic reticulum the site of the postulated intra-fibre sucrose compartment.

Dydyńska & Wilkie showed that the volume of muscle fibre water decreases in hypertonic solutions. The cells behaved approximately as perfect osmometers containing, at normal osmotic pressure, 26 % by weight (or 20 % by volume: see Blinks) of solid material. As shown in Text-fig. 2 the relationship describing the volume changes of such an osmometer does

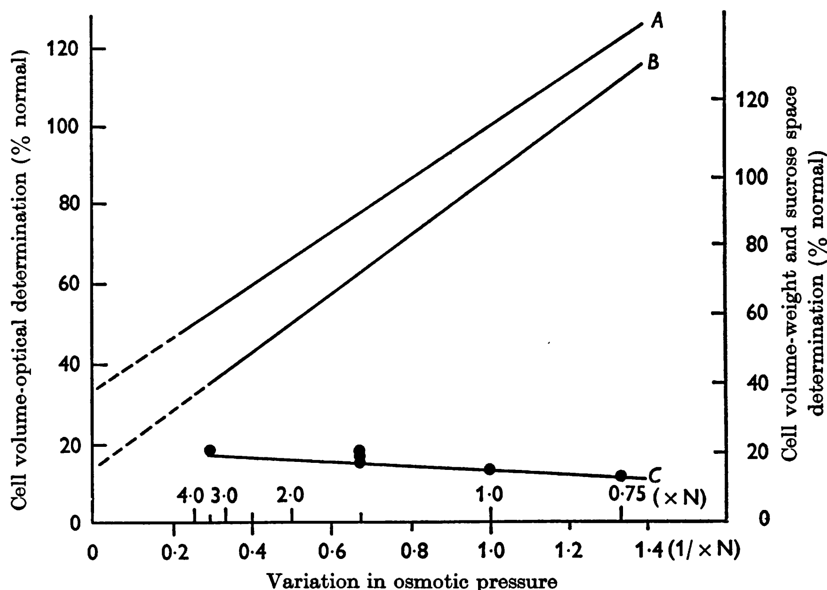


Text-fig. 2. The relationship between muscle cell volume and osmotic pressure. Line *A*, from Blinks (1965), is the line of best fit to the observed volume changes using an optical measuring technique. Line *B*, from Dydyńska & Wilkie (1963), describes the behaviour of a perfect osmometer containing solid matter equal to 20 % of cell volume at normal osmotic pressure. For further explanation see text.

not coincide with the volume behaviour of the whole fibre as measured optically by Blinks. The volume intercepts produced by the two types of measurements are, as pointed out by Blinks, grossly different, and he suggested, as had Dydyńska & Wilkie earlier, that part of this difference might be accounted for if a fraction of the sucrose space lay within the sarcoplasmic reticulum. If this hypothesis is corrrect, then by making allowance for the fact that Blinks' relationship will include the fluid volume of the sarcoplasmic reticulum, whereas Dydyńska & Wilkie's relationship will exclude it, it should be possible to predict the volume behaviour of the sarcoplasmic reticulum under different osmotic conditions.

The volume of the sarcoplasmic reticulum at normal osmotic pressure

has been estimated to be 13 % of cell volume by Peachey (1965). This estimate includes the solid material of the triad sacs, which we estimate to be 3 % of cell volume (see Methods). The fluid volume of the reticulum is therefore 10 % of cell volume. Thus Dydyńska & Wilkie's cell volume relationship should, on the basis of this hypothesis, be plotted against a scale on which 100 % corresponds with 90 % on Blinks' volume scale. By



Text-fig. 3. Comparison of the changes in volume of the sarcoplasmic reticulum determined in this study, with the changes in volume predicted by the work of Dydyńska & Wilkie (1963) and of Blinks (1965). Lines *A* and *B* from Text-fig. 2 have here been related to the left and right ordinates respectively. The two ordinates have been constructed so that 100 % on the right ordinate corresponds to 87 % on the left ordinate. The volume intercept of line *B* has been corrected to account for the solid matter of the triad sacs. For explanations see text. Line *C* is the difference $A-B$, which represents the relationship between volume of the sarcoplasmic reticulum and osmotic pressure predicted by the data of Blinks (1965), and Dydyńska & Wilkie (1963). The plotted points are the volumes of the sarcoplasmic reticulum at different osmotic pressures calculated from the reticulum dimension data presented in Table 1.

subtracting a further 3 % relative to Blinks' ordinate from the curve, to take into account the solid matter of the triad sacs, the difference between the two relationships predicts the total volume behaviour of the sarcoplasmic reticulum, and Dydyńska & Wilkie's line becomes a measure of fibre volume. The predicted volume behaviour of the sarcoplasmic reticulum is shown by the line *C* of Text-fig. 3, which indicates that the volume should be linearly related with a negative slope to the reciprocal

of the osmotic pressure. The observed behaviour of the sarcoplasmic reticulum determined in this study can now be compared with this predicted behaviour by converting our measured dimensions to volumes (see Methods). The calculated volumes are plotted in Text-fig. 3. They fit well to the predicted behaviour both with regard to the form of the relationship and with regard to absolute volume changes. The absolute volumes as calculated from our data and as predicted of course agree at normal osmotic pressure since they were taken in both cases to be the same, namely 13 % of cell volume. At 3·5 × N osmotic pressure the predicted volume is 16·6 % and the calculated volume from our data is 18·5 %. This tendency for the calculated values to exceed the predicted ones is increased slightly by distributing the zones of the reticulum to coincide more closely with the estimate of Peachey (calculated volume 18·8 %). It is also increased when the solid matter in the triad sacs is taken to be 25 % (predicted volume 15·8 %), and it is reduced when the estimate is 67 % (predicted volume 17·2 %). It is interesting to point out that in the work of Dydyńska & Wilkie the calculated fibre volumes tended, at least up to 3·5 × N Ringer, to give a smaller value than the theoretical curve we have presented from their work in Text-figs. 2 and 3. Correction of the curve to fit more closely to their measured volumes would have the effect of increasing the predicted volumes of the sarcoplasmic reticulum to match more closely our calculated points. We feel accordingly that the agreement between the predicted and the observed osmotic behaviour of the sarcoplasmic reticulum provides strong support for the view that the sarcoplasmic reticulum is accessible to sucrose, as suggested by the earlier workers, and that it may accordingly be extracellular in nature.

It is of considerable interest with regard to this hypothesis that studies on the distribution of electrolytes in frog skeletal muscle have consistently revealed a discrepancy between the actual measured intracellular ion concentrations and the concentrations which are predicted for muscle by the Donnan equilibrium. To explain this discrepancy a number of workers (Steinbach, 1947; Simon, Shaw, Bennett & Muller, 1957) have postulated the existence of a small intra-fibre compartment accessible to all extracellular ions. Harris (1963) on the basis of studies on the distribution of muscle chloride, calculated that for the Donnan equilibrium to hold, this space must contain 15 % of the total cell water (about 12 % of cell volume), a figure in remarkable agreement with the volume of the sarcoplasmic reticulum as measured by Peachey (1965), and considered as a possible location for this space by Harris. It therefore seems reasonable to look upon the reticulum space as being fundamentally extracellular in nature.

The site of continuity between extracellular space and the sarcoplasmic reticulum is suggested by electron microscopic studies on muscle. These

have shown that the transverse tubules are open to extracellular space where they make contact with the sarcolemma (Frazini-Armstrong & Porter, 1964; Huxley, 1964; Page, 1964; Birks, 1965) and it appears unlikely that any element of the sarcoplasmic reticulum itself is open to extracellular fluid at the sarcolemma. Thus the most likely site for extracellular fluid to gain access to the sarcoplasmic reticulum is at the region of contact between the transverse tubules and the triad sacs. In these regions, dense staining, apparently amorphous, bridge-like structures connect the two elements. These cross-bridges have been compared to the membrane junctions at electrically transmitting synapses (Fahrenbach, 1965) and to septate junctions joining epithelial cells (Peachey, 1965). Both of these junctions conduct electrical potential changes and they also appear to permit movement of water and small solute molecules between the connected cells, but not the movement of larger solute molecules (Lowenstein & Kanno, 1964; Potter, Furshpan & Lennox, 1966). The fact that comparison of extracellular space in frog skeletal muscle using sucrose and albumin shows a 12% smaller space for albumin (Tasker, Simon, Johnstone, Shankley & Shaw, 1959) gives striking support for the suggestion that the cross-bridges are semi-permeable pores, which provide fluid continuity between extracellular space and the sarcoplasmic reticulum (cf. Birks, 1965).

The presence within the sarcoplasmic reticulum of fluid of related composition to extracellular fluid suggests that there could well be an electrical potential difference across its limiting membranes in the same direction as exists across the sarcolemma and presumably across the T-tubule membranes. Assuming that conduction of membrane potential change can take place across the cross-bridge connexions, then during excitation the change in membrane potential, which is believed to spread from the sarcolemma to the T-tubules (Huxley & Straub, 1958; Huxley & Taylor, 1958; Strickholm, 1966), could continue directly to the triad sacs of the sarcoplasmic reticulum (Birks, 1965; Fahrenbach, 1965; Peachey, 1965), to bring about contraction through the release of calcium from these last sites (Heilbrunn & Wiercinski, 1947; Niedergerke, 1955; Hasselbach & Makinose, 1961; Ebashi & Lipmann, 1962; Weber, Herz & Reiss, 1963; Hasselbach, 1964; Podolsky, 1964; Costantin, Frazini-Armstrong & Podolsky, 1965; Winegrad, 1962*a*, *b*). This hypothesis is supported by the observation of Costantin & Podolsky (1966) and Lee, Ladinsky, Choi & Kasuya (1966) that local depolarization of the triad sacs appears to activate contraction.

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EXPLANATION OF PLATES

PLATE 1

Fig. 1. Transverse section passing mainly through the H-zone region of a frog sartorius muscle showing cross-sections of the H-zone sacs of the sarcoplasmic reticulum. The muscle was soaked for 10 min in normal Ringer (N Ringer) before fixation in acrolein. Scale bar represents 0·5 μ. All figures at same magnification.

Fig. 2. Similar view to that shown in Fig. 1 from a muscle soaked for 10 min in hypertonic Ringer ($1{\cdot}5 \times N$) before fixation in acrolein. Note dilated H-zone sacs.

PLATE 2

Fig. 1. Transverse section through the Z line and adjacent I band regions showing cross-sections of the triad sacs. The muscle was soaked for 10 min in N Ringer before fixation in acrolein.

Fig. 2. Similar view to that shown in Fig. 1 from a muscle soaked for 10 min in hypertonic Ringer ($1{\cdot}5 \times N$). Note absence of marked swelling of the triad sacs.

PLATE 3

Fig. 1. Similar view to that shown in Pl. 1, fig. 1 from a muscle soaked for 10 min in hypertonic Ringer ($3{\cdot}5 \times N$) before fixation in acrolein. Note marked swelling of the H-zone sacs.

Fig. 2. Similar view to that shown in Pl. 2, fig. 1 from a muscle soaked for 10 min in hypertonic Ringer ($3{\cdot}5 \times N$) before fixation in acrolein. Note absence of marked swelling of the triad sacs.

PLATE 4

Fig. 1. Longitudinal section showing one sarcomere of a muscle soaked for 10 min in hypertonic Ringer (3·5 × N) before fixation in acrolein. Note swelling of sarcoplasmic reticulum and persistence of continuity between the triad sacs and the H-zone sacs.

Fig. 2. Transverse section passing mainly through the H zone of a fibre, damaged as a result of soaking for 10 min in hypertonic Ringer (3·5 × N) before fixation in acrolein. Arrows indicate limits of a space probably occupied by sarcoplasmic reticulum before fixation. Note small vesicles near this space.

PLATE 5

Fig. 1. Similar view to Pl. 1, fig. 1 from a muscle soaked for 10 min in N Ringer before fixation in acrolein.

Fig. 2. Similar view to Pl. 5, fig. 1 from a muscle soaked for 10 min in hypotonic Ringer (0·75 × N) before fixation in acrolein. Note the reduction in short axis of the H-zone sacs.

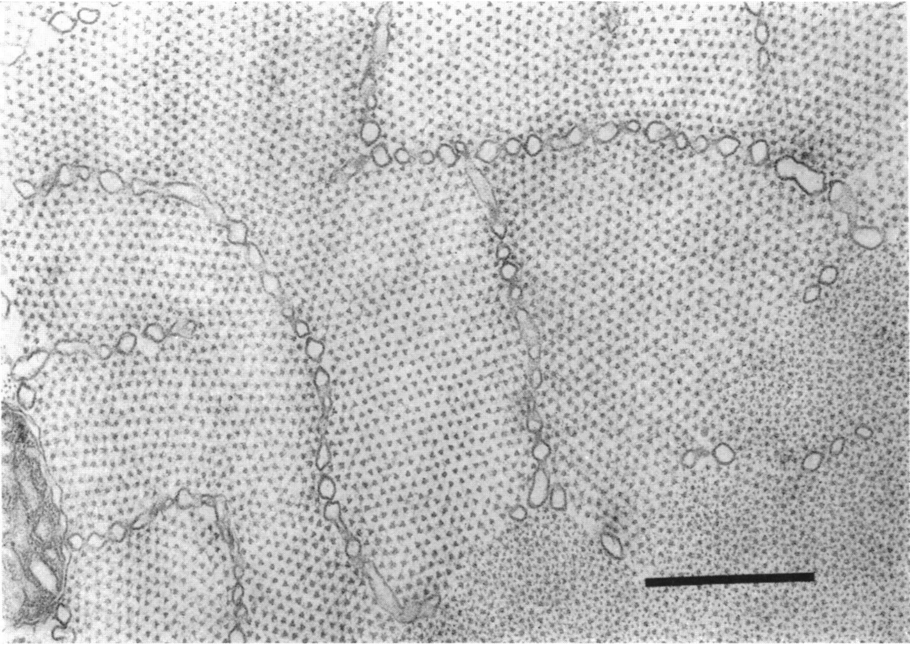
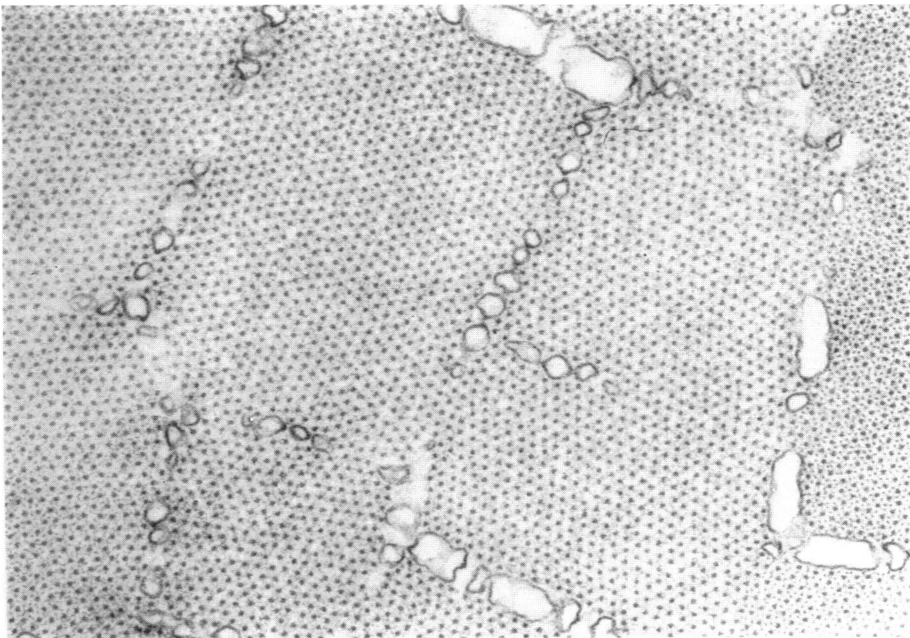


Fig. 1



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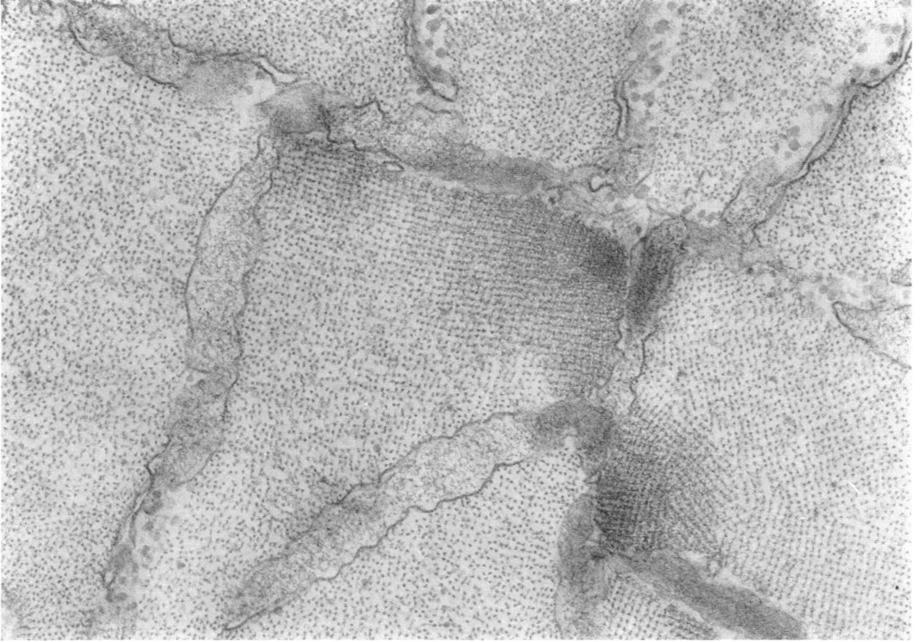

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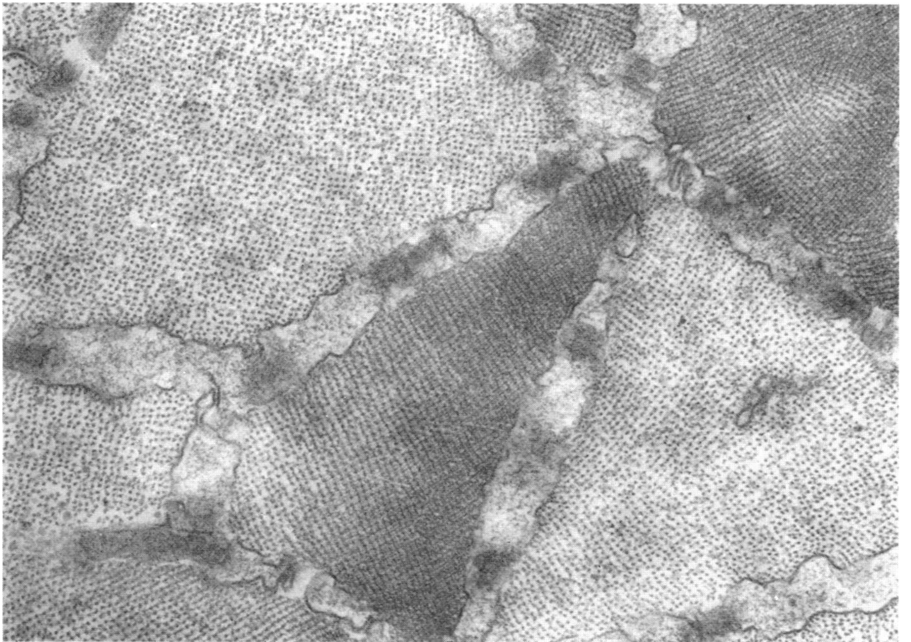

Fig. 2

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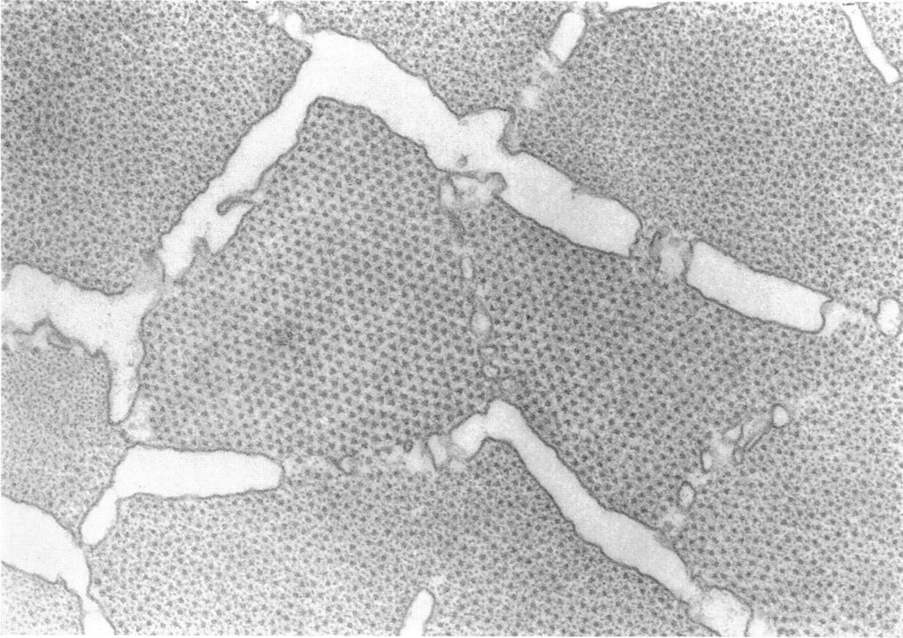

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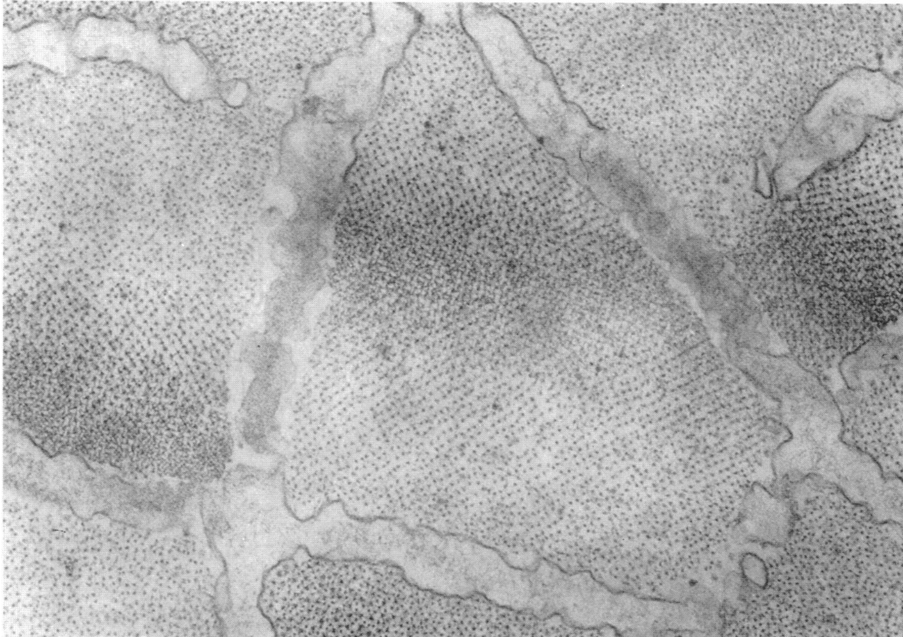

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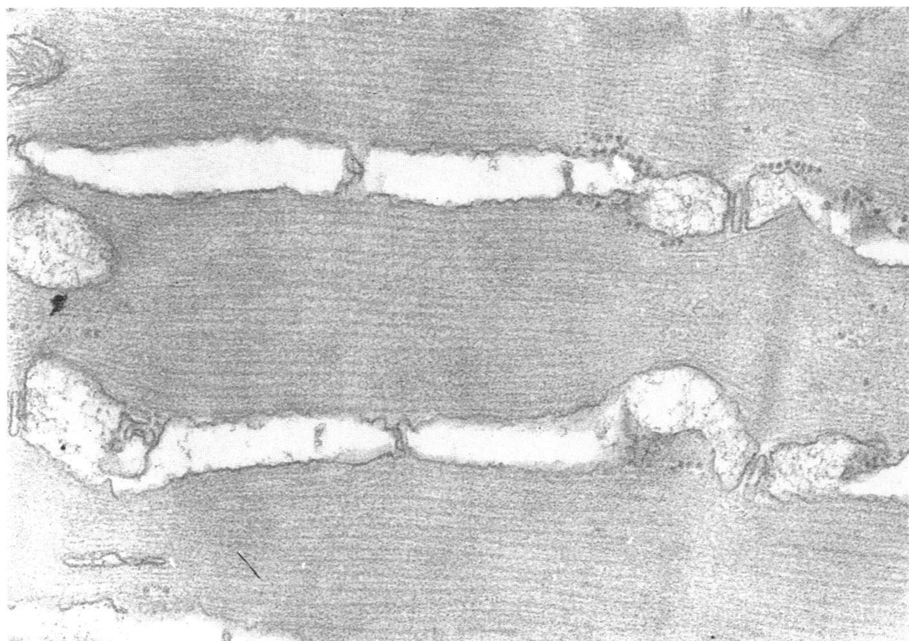

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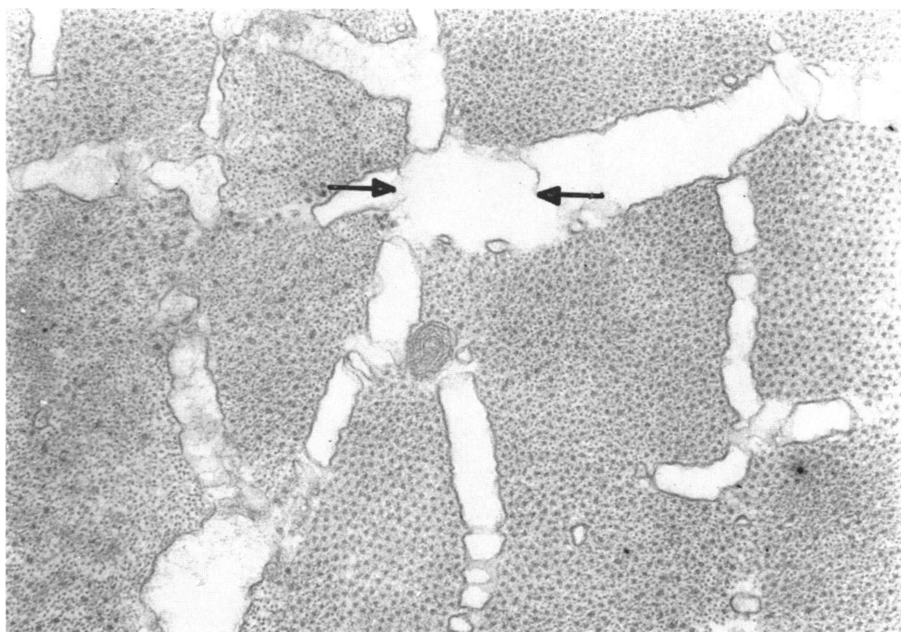

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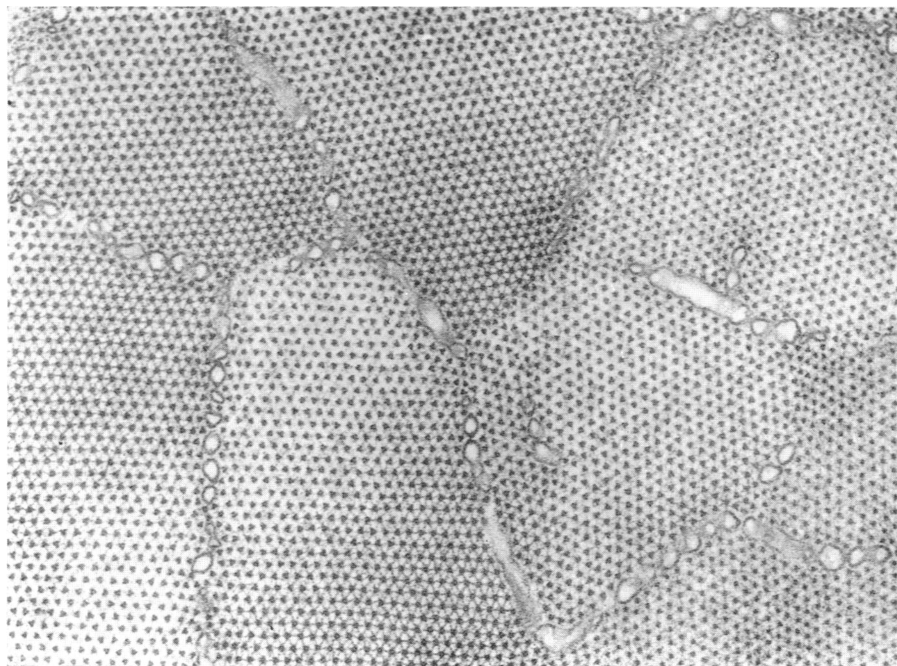

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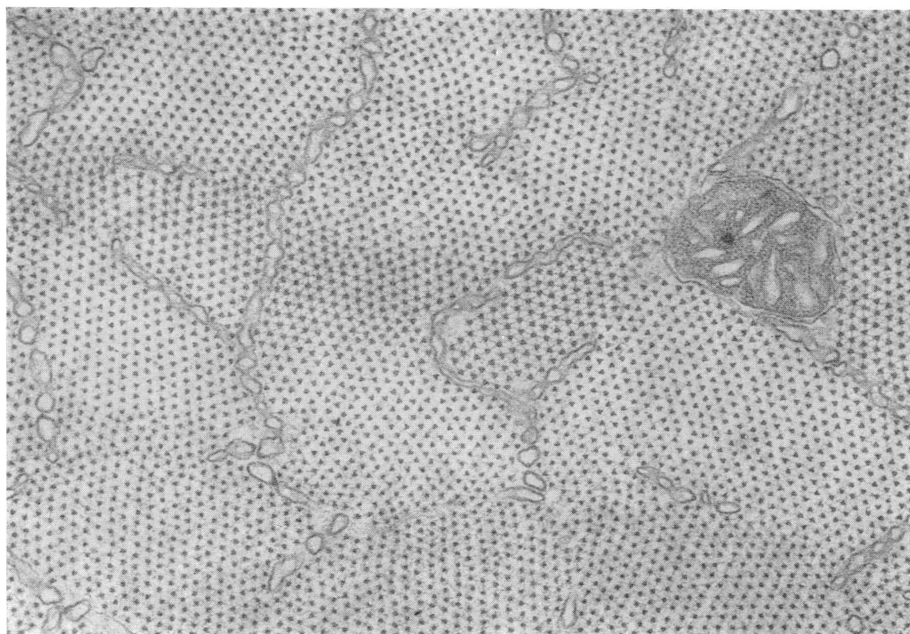

Fig. 2

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